Current Definition of Vitamin D Status Misclassifies Maladapted Children of First Generation African Immigrants to the Northern US

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Abstract

Skin pigmentation, vitamin D inactivation and genetic variation of vitamin D binding protein (DBP) are all essential mechanisms for adaptive vitamin D metabolism in African children living near the equator. The widely used measurement of total serum 25-hydroxyvitamin D (25OHD) test ignores their inherent differences and maladaptive vitamin D metabolism, and potentially misclassifies their vitamin D status in northern parts of the United States. The goal of this multi-center international cross-sectional observational study was to better define vitamin D status in Somali immigrants living in the northern US. Well children aged 6 months to 7 years from Minnesota (US-born of Somali descent, n=55) and in Uganda (n=95) were enrolled. 25OHD and other vitamin D metabolites (24,25(OH)2D) were measured by immune-affinity extraction and liquid chromatography-tandem mass spectrometry. Parathyroid hormone (PTH) and hypocalcemia status were used as indicators of insufficiency. DBP haplotypes were determined. Ninety-one percent of the Minnesota Somali participants had 25OHD levels <30 ng/mL (vs 48% in Ugandans). Compared to the Ugandan group, and despite better nutritional status (milk intake), MN Somali children had lower 25OHD (23.7 ng/mL vs 30.1; $p<0.0001$) and calcium levels (9.1 mg/dL vs 9.5; $p<0.0001$), and higher PTH levels (47 pg/mL vs 36; $p<0.0001$). Somalis had a significantly higher frequency (57% vs 14% in Ugandans; $p<0.001$) of calcium in the lower level of normal even at 25OHD levels >

20 (American Academy of Pediatrics (AAP) cutoff for sufficiency). This was not significantly different from the Somali group with 25OHD < 20 ($p<0.3$). The high affinity allele Gc1f was the predominant DBP variant in both Somalis and Ugandans, yet MN Somalis had a higher percentage of low serum calcium status. The Somali group had significantly higher levels of vitamin D inactivation (higher 24,25(OH)2D) despite having lower 25OHD levels, raising a concern of maladaptive vitamin D metabolism and inherent susceptibility to vitamin D deficiency independent of limited cutaneous vitamin D synthesis as a result of darker skin tone. These results suggest that 25OHD levels 20-30 ng/mL (above the AAP cutoff for sufficiency (>20 ng/mL)) are common in children of Somali descent in northern US, and are clinically significant. Also, while African children living near the equator possess adaptive mechanisms for acquisition and utilization of vitamin D, those same mechanisms could render them susceptible to insufficiency when migrating to high latitude regions such as the northern US.

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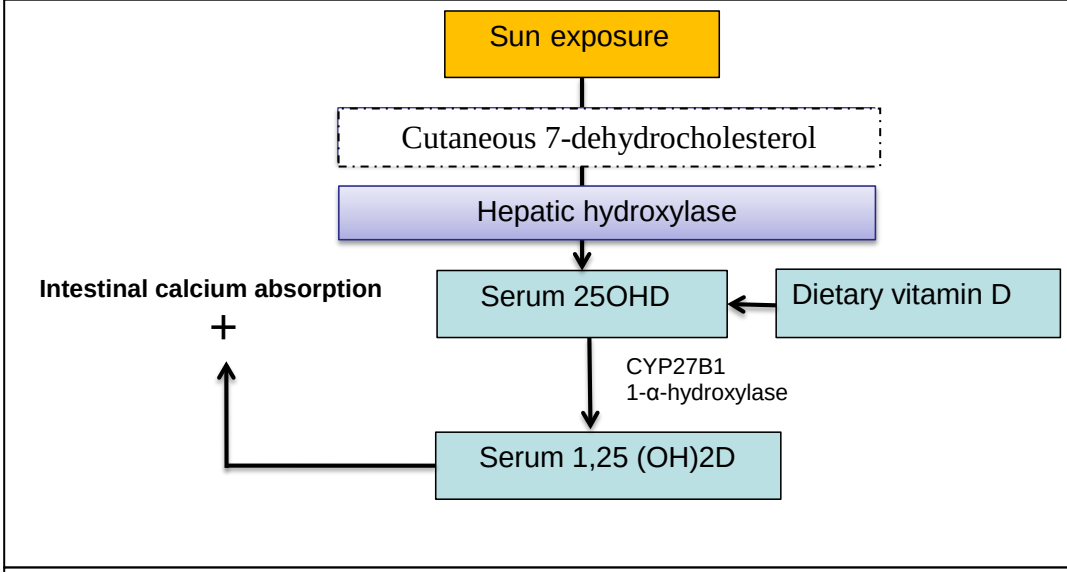
Introduction

Vitamin D, a prohormone, houses a group of fat soluble seco-sterols [1]. This essential vitamin is gained from exposure to sunlight, vitamin D rich diet as well as dietary supplements. The two main forms of vitamin D, vitamin D2 (synthesized by plants) and vitamin D3 (synthesized by animals), are found in either supplements or animal-based products, respectively [1]. Aside from the differences in chemical side-chains, previous studies have found vitamin D3 intake to be more efficient in raising serum vitamin D levels, and more effective in the human body, as opposed to D2 [2]. Nonetheless, they both still are considered to be prohormones and precursors for the biologically active vitamin D, 1,25 dihydroxyvitamin D (1,25(OH)2D). In humans, vitamin D3 can be obtained from sunlight-mediated cutaneous synthesis (figure 1).

Figure 1. Vitamin D sources and metabolism



Abbreviations: 25OHD =25-hydroxyvitamin D; 1,25(OH)2D =1,25 dihydroxyvitamin D; CYP27B1 =renal 1-α hydroxylase enzyme

Exposure to sunlight is the main source of vitamin D through the synthesis in the skin [3]. After the exposure of ultraviolet B radiation (wavelength 290 to 315 nm), the pre-vitamin compound is converted to vitamin D3 by the aide of 7-dehydrocholestrol enzyme in the skin [4]. Vitamin D undergoes hepatic hydroxylation via mitochondrial cytochrome P4502R1 (25-hydroxylase) enzyme, through a mostly unregulated catalytic step [5]. Therefore, the amount of vitamin D3 correlates with the availability of 7-dehydrocholesterol, degree of ultraviolet B radiation and duration of exposure. Other factors that can influence the body acquisition of vitamin D via the cutaneous route include: the season, the latitude, and degree of skin pigmentation [6]. Serum 25-hydroxyvitamin D levels tend to decrease during fall and winter seasons in areas with latitudes higher than 30 degrees N or S [6], especially in individuals with darker skin pigmentation or excessive use of covering and sunscreen.

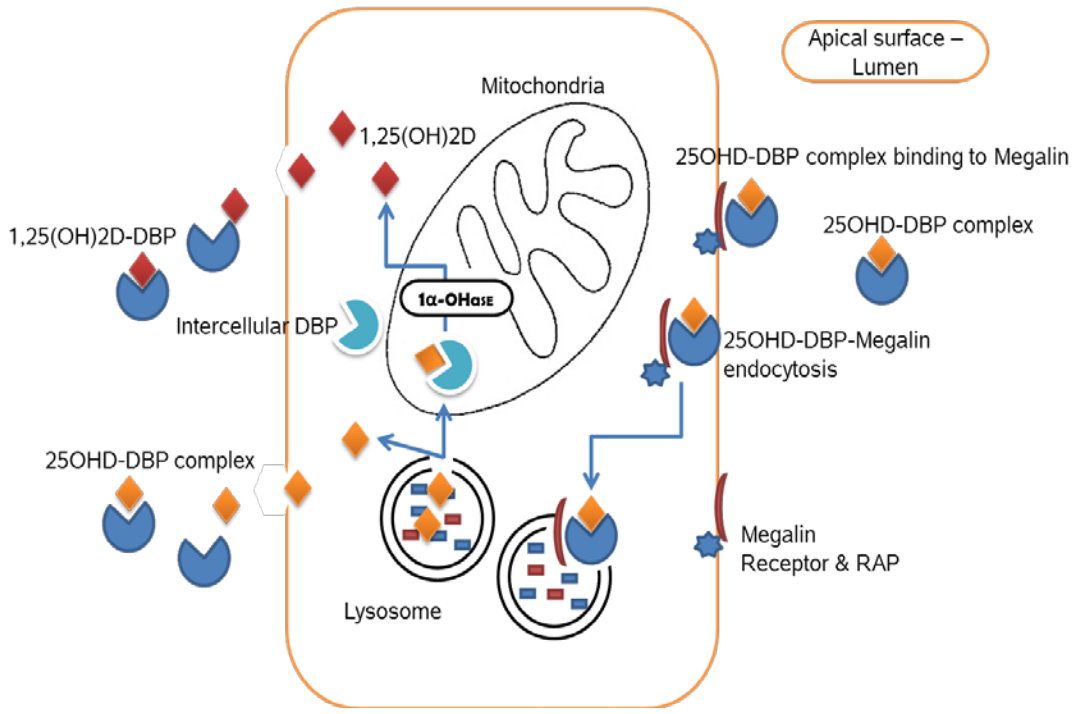
Apart from endogenously synthesized vitamin D, another source of vitamin D acquisition, to lesser degree, is the diet. The main dietary sources of vitamin D are found in vitamin D rich foods and nutritional supplements. The total dietary intake of vitamin D includes dietary intake from both supplements and food, such as fatty fish, foods fortified with vitamin D, and beef, fish, and liver. In recent years, vitamin D supplements have become more commonly used. These supplements contain either D2 or D3. The current AAP (American Academy of Pediatrics) vitamin D supplementation recommendation for infants is 400 IU daily. Based on this guideline, pediatricians typically recommend supplementation for infants who are either breastfed or receiving less than 1 liter (33.8

ounces) of infant formula a day [7]. In the US, it is recommended that liquid milk be fortified with 400 IU of vitamin D per quart but this fortification is not mandated by governmental regulations. Studies have shown that liquid milk in the US actually contains varying percentages (62% to 120%) of the recommended amount for fortification [1]. Vitamin D fortified milk and foods are important sources of vitamin D in areas at higher latitudes with diminished hours of effective sunlight.

Vitamin D, synthesized in the skin or absorbed from the diet, is transported to the liver where it is converted to 25-OHD by the CYP2R1 enzyme [1]. The newly synthesized 25-OHD binds to a vitamin D binding protein (DBP). The bound 25-OHD is hydrolyzed in the kidneys to its active form, also known as calcitriol or 1,25(OH)2D, by the CYP27B1 enzyme or alpha 1 hydroxylase. The process of vitamin D activation, 25-hydroxyvitamin D to its active form calcitriol, takes place at the renal tubular cells. Megalin receptors at the luminal sides of the cells bind the 25-hydroxyvitamin D-DBP complex to induce an endocytosis process. Intracellularly, 25-hydroxyvitamin D-DBP complex is degraded in the lysosomes before being transported by intracellular binding protein to the mitochondria where alpha 1 hydroxylase enzyme carry out the activation/conversion process (25-hydroxyvitamin D or “calcidiol” to 1,25-dihydroxyvitamin D or “calcitriol”). The intracellular processes depend on the successful megalin receptor mediated internalization of 25-hydroxyvitamin D-DBP complex (figure 2). Therefore, DBP and megalin receptors both are essential for the process. DBP transports 25-hydroxyvitamin D and in turn prevents its loss in urine, and also plays a

role in Vitamin D delivery to tubular cells' mitochondria. Megalin seems to primarily bind DBP bound 25-hydroxyvitamin D [5].

Figure 2. Renal tubular uptake of 25OHD-DBP complex and its activation



Abbreviations: 25OHD =25-hydroxyvitamin D; 1,25(OH)2D =1,25 dihydroxyvitamin D; RAP =receptor associated protein.
Adapted from Dusso, et al., American Journal of Physiology 2005

Animal studies have shown that knock-out mutations of megalin render mice susceptible to rickets when placed on a vitamin D deficient diet. Interestingly, DBP knock-out mutation mice are not typically rachitic, suggesting an alternative route of activation, but they are found to be resistant to vitamin D intoxication, emphasizing the importance of DBP role in the conversion of the precursor vitamin D form 25-hydroxyvitamin D into its active form, dihydroxyvitamin D (1,25(OH)2D) or calcitriol [5]. Parathyroid hormone

(PTH) and phosphorus and fibroblast growth factor 23 (FGF23) levels all play an important role in regulating alpha 1 hydroxylase enzyme, and thus directly influence the availability of the active form of vitamin D. When there are low levels of calcium and parathyroid hormone, the CYP27B1 enzyme is stimulated to bind and convert inactive 25-OHD to hydrolyze in the kidney (figure 1). Low serum phosphorus levels have direct and similar effects, whereas, FGF23 inhibits calcitriol production. Scientific evidence has long supported the importance of vitamin D action in skeletal bone turnover and calcium absorption.

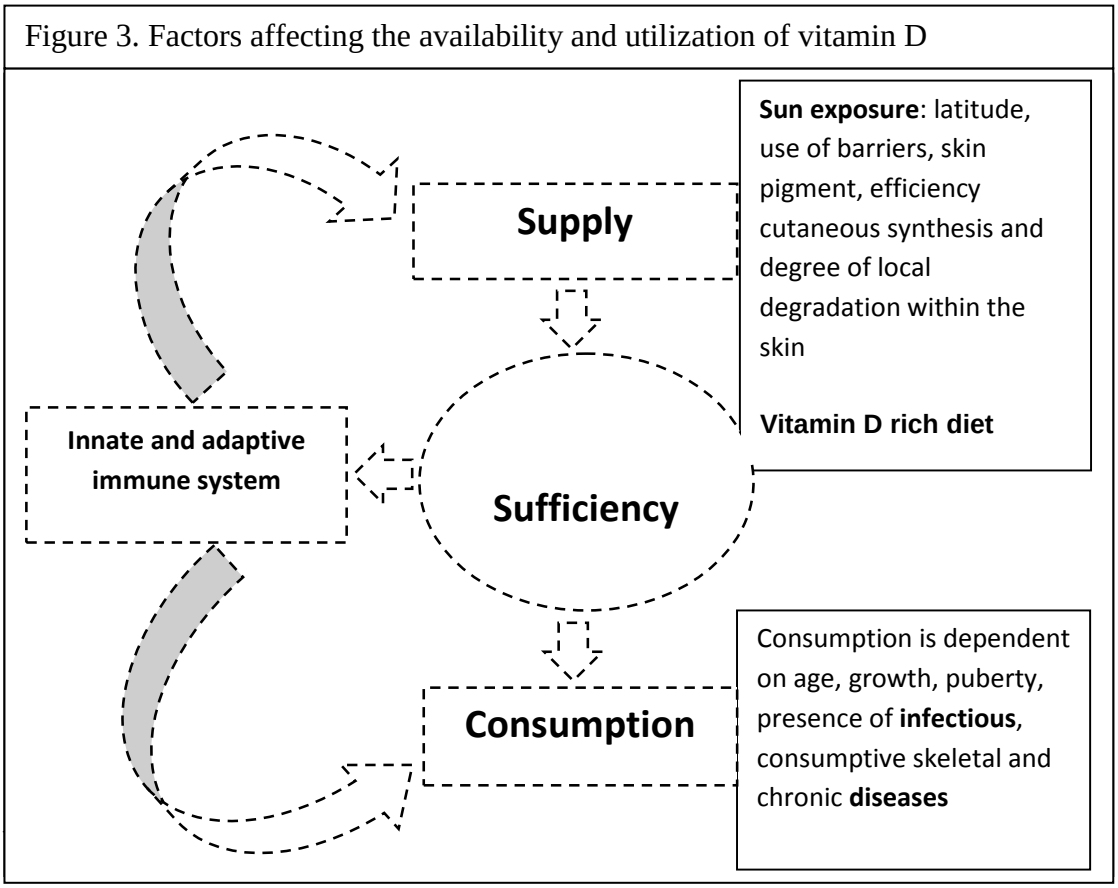
Actions of vitamin D as a hormone

At the molecular level, in humans, vitamin D exerts its action via its biologically active form 1,25(OH)2D. Vitamin D receptor (VDR) transforms into heterodimer by interacting with retinoid X receptor after binding 1,25(OH)2D. Subsequently, a response element at the nuclear level influences activation or suppression of the tissue corresponding genes to bring about induction of protein transcriptions specific for vitamin D function. If it is the intestine, 1,25(OH)2D would promote claudin 2, CaBP (calcium transporter), and TRPV6 (calcium channel), to increase calcium absorption. In case of skeletal bones, vitamin D affects both osteoblast and osteoclast via RANKL, SPP1 (osteopontin) and osteocalcin, respectively [8]. VDR are found in various tissues and organs including bone, intestine, kidneys, immunocytes (T cells), as well as gonads, brain, colon and pancreas. VDRs are also found in parathyroid glands, playing a role in decreasing PTH synthesis and secretion in state of vitamin D sufficiency [9].

Vitamin D deficiency in African children

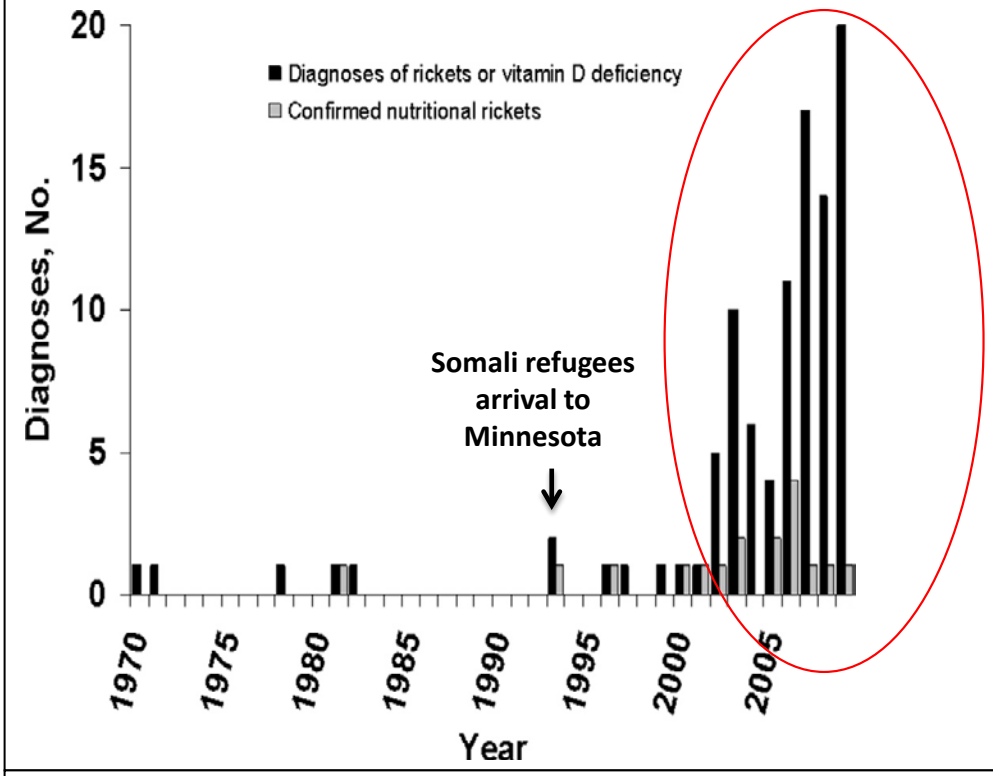
Across the African continent there are climate, latitude, and nutritional, subpopulation (within the continent) variations that influence the prevalence of vitamin D deficiency [6]. In African children, biochemical vitamin D deficiency leads to clinical rickets alone or in conjunction with dietary calcium deficiency [10]. Another major factor leading to vitamin D deficiency in Africa is the burden of infectious disease [6]. Prevalence of infectious and chronic disease can be both the cause and effect of vitamin D deficiency. The latter is proven to be related to an increased susceptibility to infections due to the known immunomodulatory role of vitamin D [11]. On the other hand, relative insufficiency of vitamin D in the context of endemic infectious disease is believed to be associated increased vitamin D consumption or over utilization (figure 3) [6].

Figure 3. Factors affecting the availability and utilization of vitamin D



A recent study in Olmsted County, Minnesota has shed light on the increased incidence of nutritional rickets in children of African descent (220 cases per 100,000). Vitamin D deficiency and nutritional rickets incidence increased in Minnesota after Somali refugees' arrival in and after 1994 (figure 4). In this study, in which 17 children had either nutritional rickets or rachitic radiological findings, the diagnosis was associated with black race (59% of all study participants with rickets out of whom majority were of Somali descent). The authors raised the possibility of dietary calcium deficiency as an explanation similar to a previous report of African children living in Africa [12].

Figure 4. Increased incidence of nutritional rickets in Minnesota



Adapted from Thacher et. al, Mayo Clin Proc. 2013

Definition of vitamin D status in African ethnicity and DBP polymorphism

Vitamin D deficiency is a worldwide problem associated with rickets, fractures, weak immunity with increased susceptibility to infection, and malignancy [4, 13]. Because of the pleiotropic effects on multiple systems, it is important to ensure optimal vitamin D levels not only to correct deficiency, but also to avoid toxicity, which can have adverse consequences as well (hypercalcemia, hypercalciuria leading to nephrocalcinosis) [1]. Typically, parameters for defining vitamin D deficiency and the need for treatment are based on total 25OHD levels [14].

However, recent studies have suggested that in some populations low total 25OHD alone does not reflect a true vitamin D deficiency status, because it does not take into account DBP affinity for vitamin D [15, 16]. It has been postulated that the portion of 25OHD which is not bound to DBP represents a “bioavailable” form of vitamin D [17]. Thus, relying on the total 25OHD alone may misclassify patients as requiring treatment if their bioavailable vitamin D levels are normal. Previous studies in African Americans have shown that blacks tend to have not only lower total vitamin D levels compared to Caucasians, but also lower DBP levels, resulting in similar concentrations of estimated bioavailable 25OHD [17]. Vitamin D circulates in the bloodstream mostly bound to DBP (85-90%). About 10-15% is bound to albumin, and only <1% is in the free form (figure 5) [15]. There are important racial differences in the genetic polymorphisms of the DBP (GC) gene that determine DBP level and affinity for vitamin D [2]. The two most common single-nucleotide polymorphisms (SNPs) in the coding region of the DBP

gene are restrictive site variants rs4588 and rs7041 [17]. Multiple studies show a significant correlation between these restriction site variants and DBP levels and affinity.

Figure 5. Vitamin D metabolites transport in the blood

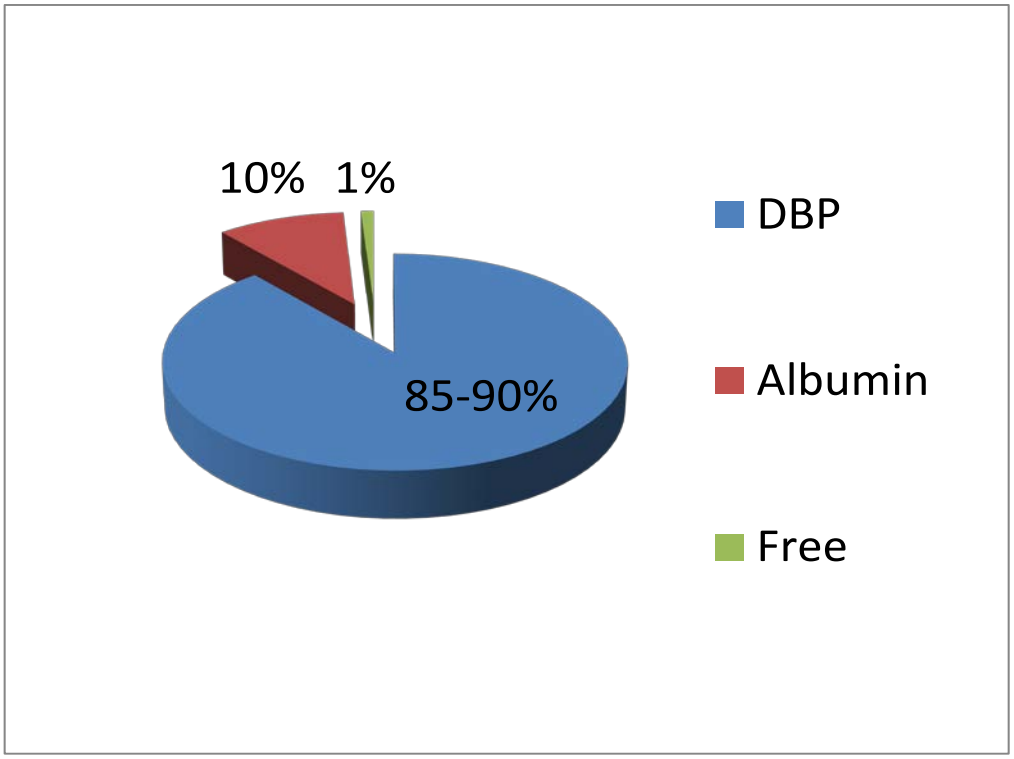


Table 1. Different national guidelines for cutoffs of vitamin D status

Vitamin D Status	Calcidiol (ng/mL)			
	AAP 2008, IOM	Endocrine Society	KDOQI	Adult – NEJM 2007
Severe deficiency	< 5	—	< 5	—
Mild to moderate deficiency	5-15	< 20	5-15	< 20
Insufficiency	16-20	21-30	16-30	20-30
Sufficiency	21-100	31-60	> 30	31-60
Excess	101-149	—	—	—
Intoxication	> 150	—	—	> 150

AAP, American Academy of Pediatrics; IOM, Institute of Medicine; KDOQI, Kidney Disease Outcomes Quality Initiative; NEJM, New England Journal of Medicine

Source: Lee et. al J Pediatric Pharmacological Therapy 2013

When considering total 25OHD levels, the cutoff between normal and abnormal levels should reflect clinical significance. Currently used cutoffs (table 1) of 25OHD levels in determining vitamin D deficiency were based on studies that utilized indicators such as radiologic changes, bone mineral density, PTH, and alkaline phosphatase levels [4]. The most sensitive biochemical indicator of vitamin D deficiency appears to be PTH level. Low levels of 25OHD are associated with higher than normal levels of PTH [18]. Research is still ongoing to clearly define the relationship between vitamin D deficiency status and PTH levels and the use of PTH as a biomarker for vitamin D biologic effect. In severe vitamin D deficiency rickets, 25 OHD is low and serum calcium and phosphorus are usually low, whereas alkaline phosphatase and PTH levels are elevated [4]. Certainly, biochemical vitamin D deficiency precedes radiological evidence of rickets. Therefore, the point of treatment is to prevent the development of florid rickets by detecting

biochemical vitamin D deficiency early. Some studies looked at free form of 25OHD as an indicator of vitamin D status

In the study by Powe et al. [17] the fraction of vitamin D not bound to DBP showed a better correlation with the level of PTH than total 25OHD. However, the authors used an immunoassay which has several limitations and a number of concerns were raised in the literature about its sensitivity, accuracy, and reliability. Importantly, polymorphisms themselves can affect antibody binding, confounding the interpretation [19]. The study by Powe et al. and its subsequent commentaries also raised many questions related to the physiological role of the bound vitamin D level [20], suggesting that it is the complex of 25OHD and DBP that is taken up by renal proximal tubular epithelial cells through receptor-mediated endocytosis, and that 25OHD within this complex is the major precursor for circulating 1,25-dihydroxyvitamin D. The latter is the active form of vitamin D that is important in the regulation of PTH levels. Thus, both total and free 25OHD appear to play a role in regulating calcium homeostasis and PTH secretion. The research focus continues to elucidate on the role of DBP as a modifier of vitamin D status and as a potential contributor to physiologic mechanisms that help humans to adapt to their endogenous climates.

Adaptive vitamin D metabolism

The widely used measurement of total serum 25-hydroxyvitamin D (25OHD) test ignores racial differences and adaptive traits, yet remains the universal screening tool of vitamin D status [13]. Studies have shown that serum 25-hydroxyvitamin D does not

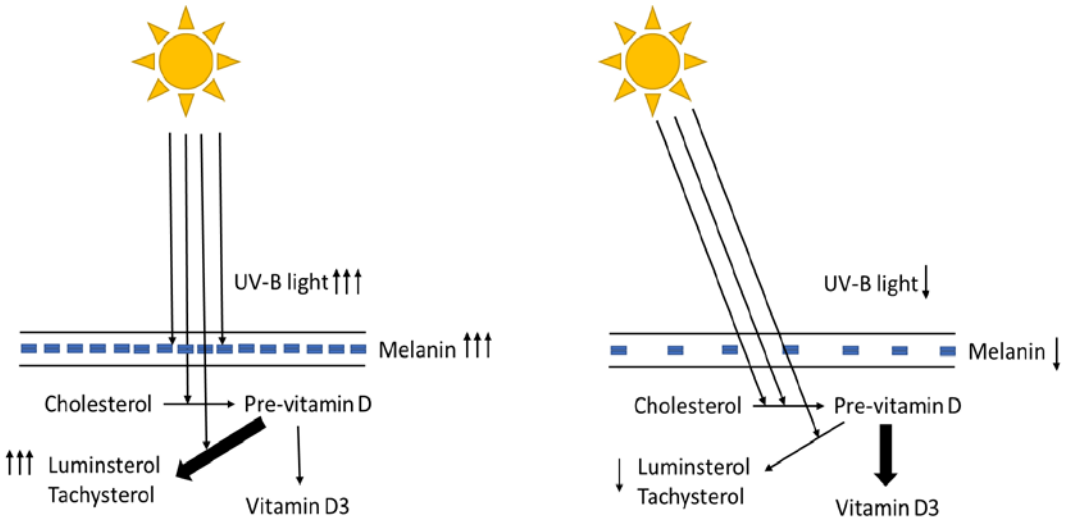
always reflect state of sufficiency especially in some ethnic populations [14].

Total serum 25-hydroxyvitamin D, which serves as a precursor [16] and substrate for the active vitamin D, 1,25(OH)2D3 and shares the same binding transport protein (DBP), variably translates into calcium absorption and skeleton accretion based on many factors that include vitamin D binding protein (DBP) phenotype and its affinity to both metabolites [13] [16]. It is certainly logical to look at general physiologic compensatory processes, which are already in place for vitamin D and calcium homeostasis, for clues to draw estimations and indicators for vitamin D status, namely serum calcium level, PTH, and alkaline phosphatase (ALP) levels. While it is established that ethnic variation in skin tones influence cutaneous synthesis of vitamin D, we are still learning about vitamin D inactivation and DBP genetic polymorphism and its distinctive racial distribution as an adaptive modifier to vitamin D status [14, 15, 20].

In central African countries like Uganda with equatorial latitudes and abundant UVB light and when nutrition is optimal, skin pigmentation, the bio-degradation of cutaneously synthesized vitamin D into lumesterol or tachysterols (which are biologically inactive), suppressed PTH and accelerated enzymatic inactivation of vitamin D are all adaptive mechanisms to counter excessive generation and utilization of vitamin D, respectively [13, 21, 22] On the other hand, it is expected to find adaptive loss of skin pigmentation and efficient cutaneous generation of vitamin D with possible diminished inactivation in indigenous inhabitants of regions farther from equator, like in northern US, Canada, and Europe, to optimize vitamin D presence and action [13, 21, 22] (figure 6 and figure 7). Other inherent adaptive mechanisms to local populations where there is

either abundance or scarcity of UVB light include, first, the inactivation pathways for vitamin D metabolite that involve CYP24 enzyme, and second, DBP polymorphism.

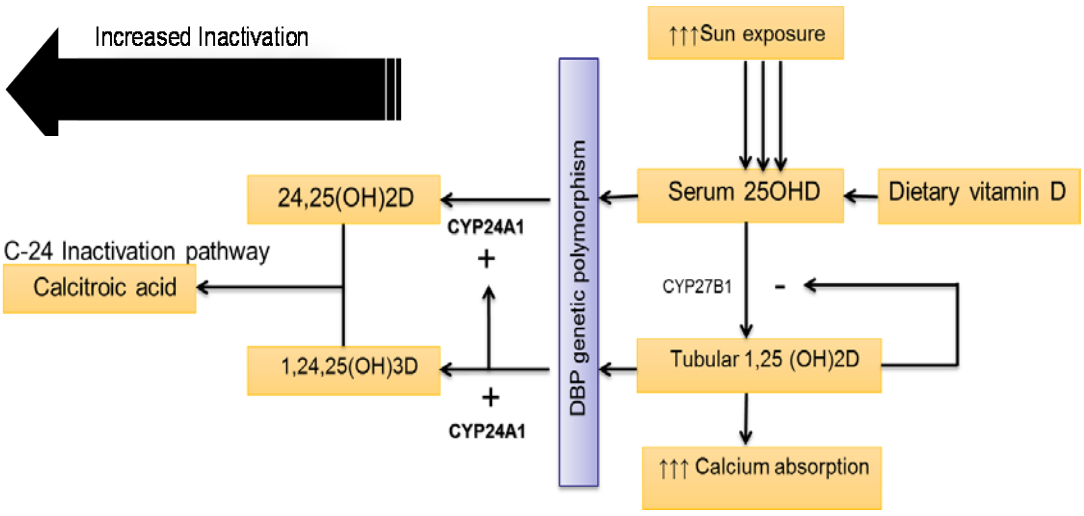
Figure 6. Vitamin D synthesis: cutaneous adaptation to various intensities of sun



Abbreviations: UV-B =ultraviolet-B

Firstly, the significance of CYP241A enzyme in vitamin D metabolism can be appreciated in the congenital infantile hypercalcemia where the deficient enzyme is associated with unregulated production of 1,25 OH2D3 [23]. It is well established that 24,25(OH)2D can correlate well with and reflect 25OHD levels because CYP241A enzyme activity is directly influenced by vitamin D receptor activity and the level of conversion product of 25OHD, 1,25 (OH)D. The relationship of the intermediate metabolite of inactivation pathway, 24,25 OH2D3, and serum calcium is as not simple as one would expect [14]. It is expected to see in vitamin D sufficiency state 24,25 OH2D3 increases and vice versa (figure 7) .

Figure 7. Post cutaneous adaptive vitamin D metabolism: increased sun exposure favors inactivation of vitamin D



Abbreviations: 25OHD =25-hydroxyvitamin D; 1,25(OH)2D =1,25-dihydroxyvitamin D; 24,25(OH)2D =24,25-dihydroxyvitamin D; CYP27B1 =1-α hydrolase enzyme; CYP24A1 =24,25 hydroxylase enzyme

Secondly, there are several studies that focused on exploring the role of the genetically determined and racially distributed DBP and whether it can significantly modify vitamin D status among different ethnic populations. Although current evidence contradicts the previous notion of ethnic difference in DBP levels in African American when compared to Caucasians in the US, the question about DBP affinity in relation to its bound and unbound vitamin D metabolite still remained unanswered. Furthermore, free 1,25(OH)2D levels appear to be well maintained even in the context of liver disease and reduced DBP levels [24]. This fact should raise speculation of the role of genetically determined DBP affinity in gauging the bioavailability of free vitamin D metabolites, 25OHD - for which there is no known receptor yet - and 1,25(OH)2D which exerts the

biological action of vitamin D including maintaining serum calcium.

It is established that a decline in extracellular ionized calcium is what triggers PTH elevation. Kruse's study described hypocalcemia (serum calcium <8.4 mg/dl) in stage 1 of deficiency in children age 2-3 months [18]. The first mechanism of maintaining serum calcium is PTH independent and relies on 1,25(OH)2D action in the intestine (particularly in duodenum and jejunum) enhancing calcium absorption [1]. Seasonal fluctuation of serum calcium between winter and summer, with the nadir observed in winters (diminished sun exposure), has been described in the literature [25]. In the absence of dietary calcium deficiency, mild subclinical hypocalcemia could be an indicator of vitamin D deficiency particularly in immigrant populations in regions farther from the equator.

In indigenous populations living in their local climates and latitudes, skin pigmentation, vitamin D inactivation and genetic DBP polymorphism seem to be essential for adaptive vitamin D metabolism. In the wake of a recent history of increased human immigration and displacement to regionswith latitudes for which they have no adaption, in addition to nutritional influences, these adaptive mechanisms may pose differential susceptibility for vitamin D insufficiency. Thus, it is conceivable that we may find in these immigrant populations vitamin D acquisition, metabolism, and physiologic compensation that may pose differential susceptibilities for insufficiency status manifested as lower 25OHD levels, mild subclinical hypocalcemia, and PTH elevation. Due to the known inter-generational genetic mix between African Americans and Caucasians and generational adaptation, we wanted to examine these research

questions and compare US children of first generation African decent to African children living in Africa. We hypothesize that:

1. Vitamin D levels 20-30 ng/ml (considered sufficient under current AAP guidelines) will be common in Somali immigrant children living in Minnesota and will be clinically significant as evidenced by calcium levels that are lower and PTH levels that are higher than established age-adjusted normal values.
2. DBP haplotypes in Somali immigrant children will be predominantly high- and intermediate-affinity
3. Ugandan children living in Uganda will have higher vitamin D levels, less hypocalcemia and lower PTH levels than the Minnesota Somali children despite a similarly low prevalence of low-affinity DBP haplotypes

In our study, where we recruited well African children in a high latitude location (MN) mostly during winter as well as African children in equatorial latitude (Uganda), our aim was to determine if the AAP defining the cutoff level for vitamin D insufficiency (<20 ng/ml) is appropriate for the children of Somali immigrants living in northern US.

Methods

Study population and design

This is a multi-center international cross sectional observational study. The study

population consisted of 150 participants. There were two recruitment centers, one in the United States and the other in Uganda. Recruitment in the city of Minneapolis enrolled 61 well US-born children of Somali descent. Additionally, a total of 99 well Ugandan children were enrolled from the city of Kampala. Local and international Institutional review board approvals were obtained and all participants' parents/caregivers provided informed consent. In Minneapolis, MN, latitude N 45, recruitment primarily took place at the community level. The majority of enrollees (n=49) had their blood drawn in the winter months of 2016, with a few participants joining the study in the spring (n=6 Somalis). In equatorial Kampala-Uganda, where children are seen for well child checks and immunizations at Mulago hospital, parents and caregivers were approached and invited for participation. Likewise, children who attended surgical clinic for otherwise minor surgical problems (e.g. hernia, hydrocele, and benign skin lesions) were also screened for enrollment. For all study groups, there were a total of 10 exclusions: 6 Somali children had inadequate blood samples and 4 Ugandan children had (after enrollment) questionable calcium intake (n=2), malaria (n=1), or malnutrition (n=1), making a total of 150 study participants: 55 Somali and 95 Ugandan. Those who gave consent and were deemed eligible participants were sent for blood draw. US and Africa samples were batched and measured in the same US laboratory for testing.

Measurements of Vitamin D and its metabolites

The University of Washington, Department of Laboratory Medicine performed vitamin D metabolite measurements. Quantification of 1,25-dihydroxy vitamin D2

[1,25(OH)2D2] 1,25-hydroxy vitamin D3 [1,25(OH)2D3], 24,25-dihydroxyvitamin D3 [24,25(OH)2D3], 25-hydroxyvitamin D2 [25OHD2], and 25-hydroxyvitamin D3 [25OHD3] in participants' serum was carried out by using immunoaffinity extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Patient serum, calibrators, and controls (400 μL) were spiked with deuterated internal standards and immunoaffinity purified using anti-1α,25-dihydroxyvitamin D beads from ALPCO. After incubation, the beads were washed and bound analytes are eluted with organic solvent. The eluent was dried down and the residue reconstituted with the derivatizing agent PTAD in acetonitrile. After incubation at room temperature, the reaction was quenched with water. A portion of the mixture was analyzed on a Waters Xevo TQ tandem mass spectrometer equipped with an Acquity UPLC. Analytes included 25(OH)D2, 25(OH)D3, 24,25(OH)2D3, 1,25(OH)2D2, and 1,25(OH)D3 with deuterated internal standards for each analyte included. Standards were prepared in stripped human serum. The ability to multiplex the analyses was facilitated by the non-specific binding of multiple vitamin D metabolites the ALPCO beads. Importantly, the beads did not bind C-3 epimers of vitamin D nor did they bind 4β,25-dihydroxyvitamin D.

Vitamin D binding protein measurement and haplotype assignment

The University of Washington, Department of Laboratory Medicine performed the quantification of vitamin D binding protein in participants' serum. Patient serum, calibrators, and controls (10 μL) were denatured, reduced, and alkylated before being proteolytically digested with trypsin. Peptides that were liberated from vitamin D binding

globulin (in addition to spiked internal standard peptides) were specifically quantified using liquid chromatography-tandem mass spectrometry and compared with a calibration curve to determine that concentration of protein in the sample. Other peptides were monitored in order to assign the polymorphisms present in the sample, which correctly assigned the genotype 97% of the time [19].

Mineral metabolism markers

Samples were obtained, immediately processed by serum separation (centrifuge), transported in dry ice and stored at −80 °C before analysis. Serum intact PTH concentration was measured using chemiluminescent immunoassay at Fairview laboratory service, West Bank Acute Care Laboratory. Serum albumin using polychromic end point methods, calcium, phosphorus, and magnesium using bichromatic endpoint, and alkaline phosphatase using bichromatic rate methods were also performed utilizing Siemen's Vista platform at Fairview laboratory service.

Data collection

After securing inform consents, participants' parents/caregivers were briefly interviewed to fill and survey questions. Subjects were screened for study eligibility. Subjects with calcium, vitamin D supplements intake, chronic liver or kidney disease, bone disease recent fracture, acute illness, on chronic medications for HIV, seizure, or glucocorticoids, history of prematurity, younger than 6 months or older than 7 years, self-identified as not Ugandan, or Somali were excluded. The subjects were surveyed for

vitamin or calcium supplementation, breastfeeding, nutritional restrictions, hours per day of sun exposure, and quantity of liquid milk intake (cups per day to generate average per week). An estimate of daily calcium intake was based on average daily milk intake (8 oz cup of cow milk contains 270 mg of calcium). Weight was measured using a well calibrated scale (local clinic scale in Uganda UNICEF SALTER ENGLAND Model 235 6S); WHO chart was used for weight and height to determine eligibility (children with weights or weights for height less than 2 SD deviation participants were excluded). Height was measured by using tape, from the base of the floor to the marked measurement on the wall.

Statistical analysis

T-test was used to compare baseline characteristics of age, BMI, PTH, ALP, vitamin D metabolites, minerals, milk, calcium intake, and hours of sun exposure. Fisher exact and Chi-square tests were used to compare proportions of gender, low calcium, and low 25OHD. Linear correlation and Pearson correlation coefficient values were generated for continuous variables. Statistical software used to carry out analysis were SAS® 9.3 and Prism GraphPad® 7.3

Results

The study population consisted of 150 participants. From the city of Minneapolis we enrolled 55 well US-born children of Somali descent (Somali group). The majority of enrollees (n=49) had their blood drawn in the winter months of 2016 (January-February-March), with few participants joined the study in the spring (n=6). A total of 95 well Ugandan children were enrolled from equatorial Kampala-Uganda.

Table 2A. Participants' characteristics

Groups (n=150)	Somalis (n=55)	Ugandans (n=95)	P-values
Age (years), mean (SE)	4.7 (0.2)	1.7 (0.2)	<0.0001
Male, no. (%)	24 (43.6)	50 (50.5)	0.4
Weight SDS, SE	1 (1.1)	0.2 (1)	<0.0001
Height SDS, SE	1.9 (1.3)	-0.2 (1.4)	<0.0001
MTV intake, no. (%)	10 (18.2)	1 (1.1)	<0.0001
Milk intake* (cup/wk), mean (SE)	15.5 (1.1)	9.4 (0.8)	<0.0001
Daily calcium intake** (mg/day), mean	>597	>361	<0.0001
Hours sun, mean (SE)	2.8 (0.4)	4.7 (0.27)	<0.0001

Abbreviations: MTV =multivitamins; SE =standard error; SDS =standard deviation score; *milk intake was underestimated in Ugandans due to breastfeeding; **calcium intake calculated based on estimated calcium content of 8 Oz (240 mL) cow milk (270 mg)

Ugandan participants were younger in age (average 1.7 years) and the majority of them were breastfed. Compared to Ugandan children, the Somali group had better nutrition in terms of milk intake (vitamin D fortified), calcium intake, and a higher percentage use of multivitamins (table 2A).

Table 2B. Participants' laboratory characteristics			
Groups (n=150)	Somalis (n=55)	Ugandans (n=95)	P-values
25OHD (ng/ml) mean (SE)	23.7 (1)	30.1 (0.8)	<0.0001
1,25 (OH)2D (pg/ml) mean (SE)	62.27 (1.5)	126 (3.5)	<0.0001
24,25(OH)2D (ng/ml) mean (SE)	1.5 (0.07)	1.15 (0.06)	<0.001
25OHD % <30 ng/ml	91	48	<0.0001
25OHD % <20 ng/ml	24	11	0.0359
Total calcium, mean (SE)*	9.1 (0.1)	9.5 (0.04)	<0.0001
Low serum calcium (%)**	51.9	13.7	<0.0001
Phosphorus, mean (SE)	4.7 (0.1)	4.9 (0.06)	<0.01
Magnesium, mean (SE)	2.1 (0.02)	2.3 (0.02)	<0.0001
PTH (pg/ml), mean (SE)	47 (3)	35.8 (2)	<0.01
ALP, (U/L) mean (SE)	263 (14)	302.4 (11)	0.081
High/medium affinity DBP haplotype %	82	77	0.5
Low Affinity DBP haplotype %	18	23	0.45
ALP =alkaline phosphatase; PTH =intact parathyroid hormone; 25OHD =total 25-hydroxyvitamin D 2&3; 1,25(OH)2D =total 1,25-dihydroxyvitamin D 2&3; 24,25(OH)2D =total 24,25-dihydroxyvitamin D 2&3 DBP =vitamin D binding protein; *total serum calcium and corrected for albumin; **low serum calcium (total, albumin corrected), low for age and laboratory reference (<8.5 mg/ml for age <1 year and <9.1 for age >1 year)			

More than 90% of Somali immigrant children living in Minnesota had 25OHD levels less than 30 ng/ml. This was associated with a high prevalence of calcium levels below the lower limit of normal for age, and lower minerals averages (phosphorus, and magnesium), compared to Ugandan children (table 2B). Vitamin D levels <30 ng/ml were much less common in children living in Uganda, but for those with 25OHD levels <30, the prevalence of calcium levels below the lower limit of normal and PTH levels were lower than in Minnesota Somali children. Ugandans, who live at the equator, had

significantly higher hours of sun exposure (table 2A).

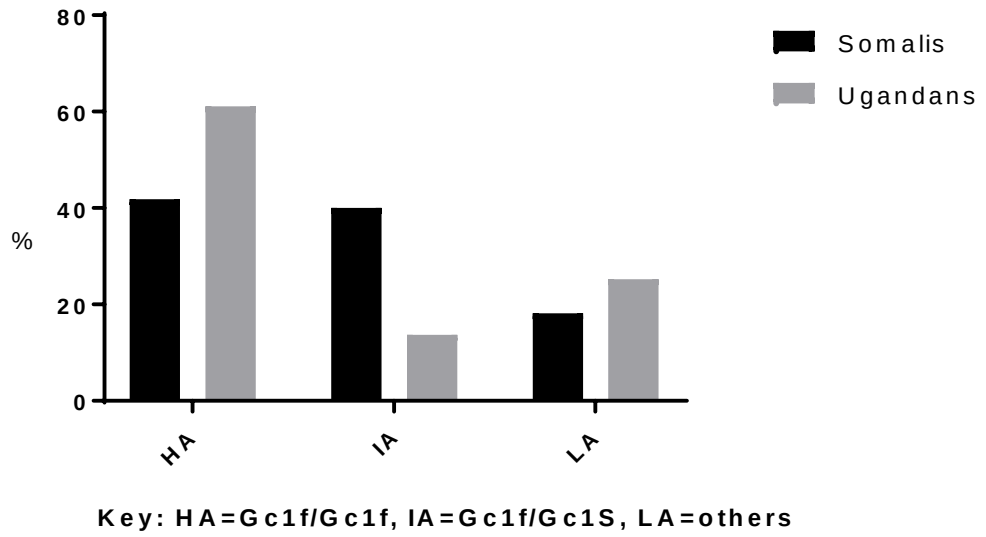
Using the cutoff of insufficiency defined as 25OHD level less 30 ng/ml (Endocrine society guideline), 49% of Ugandan children living in Africa at the equator had vitamin D insufficiency with only 13.7% (out of all Ugandan group) with low calcium levels. Whereas, percentage vitamin D insufficiency – based on the same cutoff – in Somali Minnesotans was 91% with 52% of them (out of all Somali group) having low calcium levels. In spite of adequate daily calcium intake (average 600 mg/day calcium from milk alone) and even at 25OHD levels above 20 (AAP cutoff for sufficiency), a significant percentage of participants had low calcium for age per laboratory reference values; Among Somalis with 25OHD levels less than 20 ng/ml (n=13), their hypocalcemia percentage was 38.5%, not significantly different from 56% for those (n=37) with 25OHD level less than 30 but higher than 20 ng/ml (table 3).

Table 3. Metabolic indicators for similarly low 25OHD levels in Somalis and Ugandans

	Somalis (n=55)		Ugandans (n=95)	
	25OHD cut-off levels (ng/ml)		25OHD cut-off levels (ng/ml)	
	(20-30)	<20	(20-30)	<20
N	37	13	36	10
Average 25OHD (ng/ml)	24	17	25	16
Average serum calcium, mg/dl	9	9.1	9.5	9.5
%Ca<LLN	57	39	17	10
Average PTH (pg/ml),	48	47	37	48

N = number; PTH =intact parathyroid hormone; 25OHD =25-hydroxyvitamin D, LLN =lower limit of normal =serum calcium (total, albumin corrected) low for age and laboratory reference

Figure 8. DBP haplotypes distribution



HA =affinity; IA =intermediate or medium affinity; LA =low affinity binding protein haplotypes. Gc1f and Gc1S are vitamin D binding protein (*Gc*) gene variants

Both of the groups DBP haplotype consisted of Gc1f as the predominant allele. Both Somali and Ugandan children had DBP haplotypes which were predominantly high and intermediate affinity, with ≤25% low affinity DBP haplotypes (figure 8). The children of Somali descent group tended to have a higher percentage of low calcium for laboratory reference when they possessed the high affinity haplotype (Gc1f/Gc1f) as compared to those possessing the intermediate affinity haplotype (69.5 % vs 40.9%, *p*-value = 0.05) (table 4).

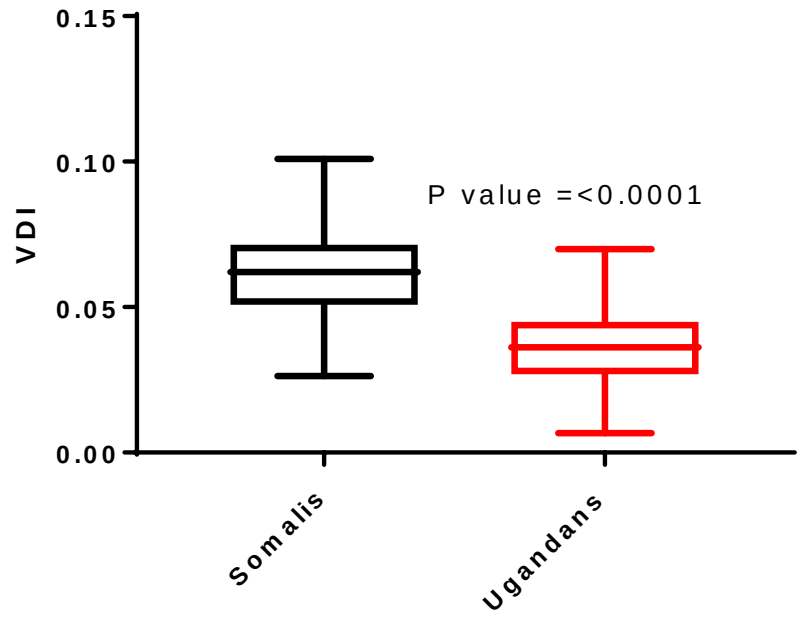
Table 4. Within group comparison of vitamin D metabolism indicators by DBP haplotype: high affinity (Gc1f/Gc1f) vs intermediate affinity (Gc1f/Gc1s)						
	Somalis			Ugandans		
DBP haplotype	Gc1f/Gc1f (n=23)	Gc1f/Gc1s (n=22)	P-value	Gc1f/Gc1f (n=58)	Gc1f/Gc1s (n=13)	P-value
PTH, mean (SE)	47.1 (18.5)	47.2 (18.5)	0.1	33.6 (24)	38.4 (24)	0.4
25OHD, mean (SE)	23.4 (6.7)	24.8 (4.6)	0.4	31.1 (9.1)	30.85 (9)	0.9
% 25OHD<30	87%	91%	0.6	40%	38%	0.9
% low calcium*	70%	40%	0.05	16%	8%	0.5
Gc1f and Gc1S are vitamin D binding protein (Gc) gene variants; PTH =intact parathyroid hormone; 25OHD =25-hydroxyvitamin D; * =serum calcium (total, albumin corrected) low for age and laboratory reference						

After adjusting for milk intake and BMI, 25OHD and PTH levels comparison between the two groups by haplotypes, (high affinity Gc1f/Gc1f vs intermediate affinity Gc1f/Gc1s, only variants with enough sample size to compare), showed that, unlike intermediate affinity, higher affinity haplotype levels of 25OHD differed significantly between groups with Ugandans once again having higher levels of 25OHD and lower levels of PTH (table 5).

Table 5. Vitamin D metabolism indicators compared by haplotypes				
High affinity haplotype (Gc1f/Gc1f)			Intermediate Affinity haplotype (Gc1f/Gc1s)	
Ugandans (n=58) minus Somalis (n=23)			Ugandans (n=13) minus Somalis (n=22)	
	Not adjusted	Adjusted *	Not adjusted	Adjusted*
25OHD, Estimate (SE)	7.7 (1.8)	8.8 (2.1)	6.1 (2.6)	7.23 (2.8)
P-value	<0.0001	<0.0001	0.02	0.01
PTH, Estimate (SE)	-13.5 (4.9)	-11.2 (5.5)	-8.8 (6.9)	-8.0 (7.4)
P-value	0.006	0.045	0.205	0.28
*Adjusted for milk intake (US milk is vitamin D fortified); 25OHD =25-hydroxyvitamin D; PTH =parathyroid hormone				

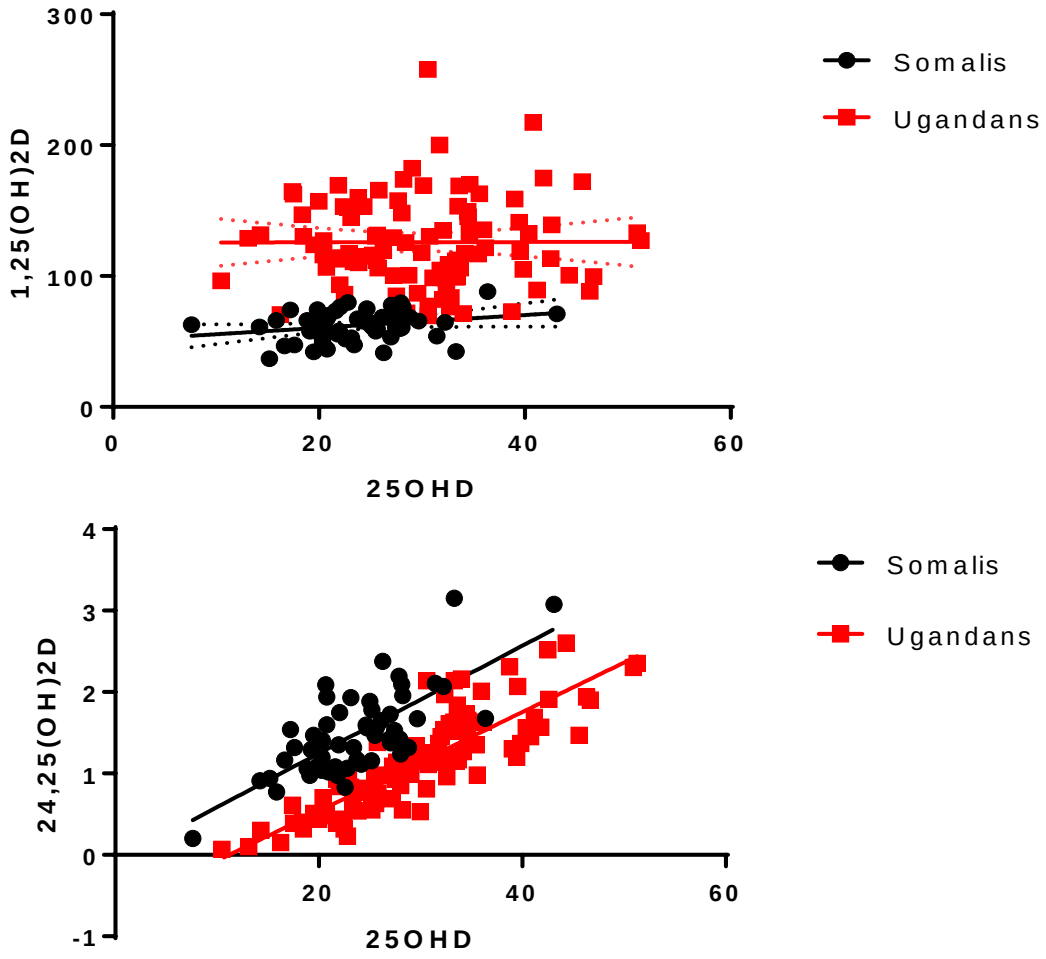
Despite the observation that Ugandan children had much higher 25OHD, 1,25(OH)2D levels and low PTH, indicating less overall vitamin D insufficiency, they had on the other hand a much lower 24,25(OH)2D compared to Somali children (figure 9). We found a clear strong and positive correlation between 24,25(OH)2D and 25OHD in both groups but not with serum calcium; the Somali group had distinctively higher levels of vitamin D inactivation (24,25(OH)2D) relative to 25OHD levels (figure 10).

Figure 9. Vitamin D inactivation relative to 25OHD level



Abbreviations: 24,25(OH)2D =24,25-dihydroxyvitamin D; 25OHD =25-hydroxyvitamin D; VDI = the ratio between participants' 24,25(OH)2D relative to 25OHD level

Figure 10. For a given 25OHD level, Minnesota Somali children had dramatically less 1,25(OH)2D and more 24,25(OH)2D



Abbreviations: 25OHD =25-hydroxyvitamin D; 1,25(OH)2D =1,25-dihydroxyvitamin D; 24,25(OH)2D =24,25-dihydroxyvitamin D

Discussion

25OHD level as an indicator of vitamin D status

Measurement of serum total 25OHD level remains a widely used screening tool for vitamin D status. In our study, we demonstrated that despite relatively better nutrition (intake of fortified milk, calcium and vitamin D supplementation) in the Somali group, we observed lower vitamin D and minerals levels (calcium, phosphorus and magnesium) and higher PTH levels compared to the Ugandan group, where higher 25OHD and minerals levels and lower PTH levels were observed. It is clear that UV-B exposure qualitatively (equatorial latitude) and quantitatively (number of hours of sun exposure) outweighs nutrition as a determinant of 25OHD level. With the marked availability of vitamin D supplementation and the emergence of evidence establishing associations between vitamin D deficiency and other non-skeletal health outcomes, it has become increasingly crucial to accurately define deficiency to allow prompt and appropriate treatment and avoid over-diagnosis and potential over treatment complications like vitamin D toxicity and nephrocalcitonosis. Despite the reported impaired calcium absorption and negatively affected bone mineral density that prompted changing the lower limit of insufficiency cutoff in adult to 30 ng/ml (ENDO guidelines), current guidelines for defining insufficiency in children remain at <20 ng/ml awaiting new research in infant and children [4]. In our study, if current AAP vitamin D insufficiency guidelines (<20 ng/ml) were to be applied to US children of Somali decent, with the

incidence of hypocalcemia and elevated PTH as a true outcome of insufficiency, a significant portion of them would be misclassified as having adequate levels of vitamin D. While this cutoff may be appropriate for Caucasian children or African Americans who underwent generational genetic mix and perhaps adaptation, it seems inappropriate in diagnosing insufficiency in children of early generation African immigrants.

Mild hypocalcemia as an outcome of vitamin D deficiency

Having statistically significant different average PTH levels between the high vitamin D high calcium group (Ugandans) and the mostly low vitamin D low calcium group (Somalis) indicates the physiologic significance of such a difference in serum total calcium (mean difference of 0.4 mg/dl). Although the relatively small percentage of hypocalcemia in the Ugandan group can be attributed to deficient calcium intake and not merely vitamin D deficiency or a combination of both, hypocalcemia in the Somali group must have been in association of vitamin D deficiency as their reported calcium intake was adequate based on current guidelines for dietary calcium [1]. One of the limitations to this assessment, however, includes the lack of knowledge about participants' ionized calcium status. We also do not have an estimate of hypocalcemia effect on bone mineral density in study participants.

Vitamin D binding protein genetic polymorphism and vitamin D inactivation

This study illustrated the distribution of different variants of DBP with an attempt to understand its impact in modifying vitamin D status. Both the Somalis and the

Ugandan group had Gc1f as the predominant allele. The Ugandan group had slightly higher percentage of homozygous high affinity Gc1f allele, but they both consistently exhibited low percentages of low-affinity alleles. This observation may be consistent with the difference in ancestral origins of both groups. Although we did not observe a direct modifying effect in 25OHD-PTH relation, there were some interesting findings. As a group, Ugandans had higher vitamin D levels and lower PTH when compared to the Somalis. At the level of haplotype affinities, the difference in average PTH and 25OHD levels between Somalis minus Ugandans is maintained for high affinity participants (Gc1f/Gc1f) but gets attenuated for the lower affinity (Gc1f/Gc1s). It seems that high affinity participants have tendency to be susceptible to vitamin D insufficiency. It is possible that this high affinity DBP allele is part of the equatorial adaptive vitamin D metabolism to the abundance and excessive synthesized 25OHD from sun light where vitamin D action is somewhat curbed by a tighter affinity thus diminishing bioavailable active vitamin D from exerting excessive calcium absorption. It is possible that for 25OHD, high affinity DBP prevents urinary loss and drives tubular endocytosis of DBP-25OHD complex for activation leading to high intracellular total 1,25OH2D that similarly unable to escape the tighter affinity after joining the DBP in the blood stream. We wonder if higher intra-tubular cells 1,25(OH)D in this haplotype could trigger relatively more mitochondrial enzyme CYP241, an activity attributing to the observed efficient inactivation pathways for both metabolites (25OHD and 1,25(OH)D).

It is unclear of how DBP variants with different affinities affect the catalytic

capacity of inactivation enzyme CYP241A. It was clearly observed in the Somali group - who had an overall similar DBP variants to the Ugandans – that they possessed high inactivation metabolite 24,25(OH)2D levels, indicating another unknown genetic difference between the two populations. Somali ancestrally come from Nile-Saharan or African-Asian who are typically exposed directly to excessive intense sun light, whereas Ugandans descend from Africa Southeastern Bantu ethnicity that inhabited the shaded jungles of equatorial Africa. Do Somali children living in Somali have similar vitamin D metabolism to Minnesota children of Somali immigrants? And could immigrant Ugandans living in northern or southern latitudes have similar increased inactivation phenomena, suggesting unknown environmental influence? Further research is needed to further elucidate these interesting scientific questions

Conclusions

While well-nourished African children at the equator possess adaptive mechanisms for acquisition and utilization of vitamin D such as darkly pigmented skin and genotypes that favor high affinity vitamin D binding (reducing availability of active free vitamin D), those same mechanisms can render them susceptible to insufficiency when migrating to high latitude regions like in northern US.

The current American Academy of Pediatrics cutoff level of insufficiency of serum 25OHD at < 20 ng/ml, may not be appropriate to apply to this population. Because of

lower calcium and higher PTH levels in Minnesota Somali children with vitamin D levels <30 ng/ml, supplementation should be offered to these children. Ugandan children use vitamin D more efficiently, with greater conversion of 25OHD to 1,25(OH)2D and less inactivation via 24, 25(OH)2D than Somali children. This is likely due to the genetic differences between the populations which make Somalis even more susceptible to vitamin D deficiency when they move away from the equator. Further research is needed to explore the observed unusually high degree of vitamin D inactivation in Somali children

Bibliography

1. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, DurazoArvizu RA, Gallagher JC, Gallo RL, Jones G *et al*: **The 2011 Report on Dietary Reference Intakes for Calcium and Vitamin D from the Institute of Medicine: What Clinicians Need to Know**. *The Journal of clinical endocrinology and metabolism* 2011, **96**(1):53-58.
2. Trang HM, Cole DE, Rubin LA, Pierratos A, Siu S, Vieth R: **Evidence that vitamin D3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D2**. *The American journal of clinical nutrition* 1998, **68**(4):854-858.
3. Wagner CL, Greer FR: **Prevention of rickets and vitamin D deficiency in infants, children, and adolescents**. *Pediatrics* 2008, **122**(5):1142-1152.
4. Misra M, Pacaud D, Petryk A, Collett-Solberg PF, Kappy M: **Vitamin D Deficiency in Children and Its Management: Review of Current Knowledge and Recommendations**. *Pediatrics* 2008, **122**(2):398-417.
5. Dusso AS, Brown AJ, Slatopolsky E: **Vitamin D**. *American journal of physiology Renal physiology* 2005, **289**(1):F8-28.
6. Prentice A, Schoenmakers I, Jones KS, Jarjou LMA, Goldberg GR: **Vitamin D Deficiency and Its Health Consequences in Africa**. *Clinical Reviews in Bone and Mineral Metabolism* 2009, **7**(1):94-106.
7. Perrine CG, Sharma AJ, Jefferds MED, Serdula MK, Scanlon KS: **Adherence to Vitamin D Recommendations Among US Infants**. *Pediatrics* 2010, **125**(4):627.
8. Haussler MR, Whitfield GK, Kaneko I, Haussler CA, Hsieh D, Hsieh JC, Jurutka PW: **Molecular mechanisms of vitamin D action**. *Calcif Tissue Int* 2013, **92**(2):77-98.
9. Bouillon R, Van Cromphaut S, Carmeliet G: **Intestinal calcium absorption: Molecular vitamin D mediated mechanisms**. *Journal of cellular biochemistry* 2003, **88**(2):332-339.
10. Thacher TD, Fischer PR, Pettifor JM, Lawson JO, Isichei CO, Reading JC, Chan GM: **A Comparison of Calcium, Vitamin D, or Both for Nutritional Rickets in Nigerian Children**. *New England Journal of Medicine* 1999, **341**(8):563-568.
11. Aranow C: **Vitamin D and the Immune System**. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research* 2011, **59**(6):881-886.
12. Thacher TD, Fischer PR, Tebben PJ, Singh RJ, Cha SS, Maxson JA, Yawn BP: **Increasing Incidence of Nutritional Rickets: A Population-Based Study in Olmsted County, Minnesota**. *Mayo Clinic proceedings Mayo Clinic* 2013, **88**(2):176-183.
13. Fuleihan Gel H, Bouillon R, Clarke B, Chakhtoura M, Cooper C, McClung M, Singh RJ: **Serum 25-Hydroxyvitamin D Levels: Variability, Knowledge Gaps, and the Concept of a Desirable Range**. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 2015, **30**(7):1119-1133.

14. Berg AH, Powe CE, Evans MK, Wenger J, Ortiz G, Zonderman AB, Suntharalingam P, Lucchesi K, Powe NR, Karumanchi SA *et al*: **24,25dihydroxyvitamin D(3) and Vitamin D Status of Community Dwelling Black and White Americans**. *Clinical chemistry* 2015, **61**(6):877-884.
15. Pekkinen M, Saarnio E, Viljakainen HT, Kokkonen E, Jakobsen J, Cashman K, Makitie O, Lamberg-Allardt C: **Vitamin D binding protein genotype is associated with serum 25-hydroxyvitamin D and PTH concentrations, as well as bone health in children and adolescents in Finland**. *PloS one* 2014, **9**(1):e87292.
16. Thacher TD, Clarke BL: **Vitamin D Insufficiency**. *Mayo Clinic proceedings* 2011, **86**(1):50-60.
17. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, Tamez H, Zhang D, Bhan I, Karumanchi SA *et al*: **Vitamin D-binding protein and vitamin D status of black Americans and white Americans**. *The New England journal of medicine* 2013, **369**(21):1991-2000.
18. Kruse K: **Pathophysiology of calcium metabolism in children with vitamin Ddeficiency rickets**. *The Journal of pediatrics* 1995, **126**(5 Pt 1):736-741.
19. Henderson CM, Lutsey PL, Misialek JR, Laha TJ, Selvin E, Eckfeldt JH, Hoofnagle AN: **Measurement by a Novel LC-MS/MS Methodology Reveals Similar Serum Concentrations of Vitamin D-Binding Protein in Blacks and Whites**. *Clinical chemistry* 2016, **62**(1):179-187.
20. Hollis BW, Bikle DD: **Vitamin D-binding protein and vitamin D in blacks and whites**. *The New England journal of medicine* 2014, **370**(9):879-880.
21. Hollis BW: **Circulating 25-hydroxyvitamin D levels indicative of vitamin D sufficiency: implications for establishing a new effective dietary intake recommendation for vitamin D**. *The Journal of nutrition* 2005, **135**(2):317-322.
22. Jacobs ET, Van Pelt C, Forster RE, Zaidi W, Hibler EA, Galligan MA, Haussler MR, Jurutka PW: **CYP24A1 and CYP27B1 Polymorphisms Modulate Vitamin D Metabolism in Colon Cancer Cells**. *Cancer research* 2013, **73**(8):2563-2573.
23. Schlingmann KP, Kaufmann M, Weber S, Irwin A, Goos C, John U, Misselwitz J, Klaus G, Kuwertz-Bröking E, Fehrenbach H *et al*: **Mutations in CYP24A1 and Idiopathic Infantile Hypercalcemia**. *New England Journal of Medicine* 2011, **365**(5):410-421.
24. Bikle DD, Gee E, Halloran B, Haddad JG: **Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease**. *Journal of Clinical Investigation* 1984, **74**(6):1966-1971.
25. **Optimal calcium intake. National Institutes of Health**. *Connecticut medicine* 1994, **58**(10):613-623.